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Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes

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Dendritic cells (DCs) are professional antigen presenting cells with the unique capacity to induce primary and secondary immune responses *in vivo*. Here, we show that DCs secrete antigen presenting vesicles, called exosomes, which express functional Major Histocompatibility Complex class I and class II, and T-cell costimulatory molecules. Tumor peptide-pulsed DC-derived exosomes prime specific cytotoxic T lymphocytes *in vivo* and eradicate or suppress growth of established murine tumors in a T cell-dependent manner. Exosome-based cell-free vaccines represent an alternative to DC adoptive therapy for suppressing tumor growth.

The challenging goal of vaccination in cancer-bearing patients is to induce long-lasting antigen-specific immunity to protect the host against tumor establishment. To date, active specific immunotherapy for cancer relies on the use of intact native proteins, cytotoxic T-lymphocyte (CTL)-defined peptides, nucleic acids and recombinant viruses, as well as genetically modified-tumor or antigen presenting cells[1–4]. Interestingly, the dominant role of bone marrow-derived host APCs in CTL induction *in vivo* has been demonstrated following most of these immunizations[5,6].

Although the *in vivo* relevance of DCs[7–11] in antitumor immune responses has not yet been formally established, correlations between cancer patient prognosis and DC infiltrates in tumor specimen have been described by pathologists[12]. Importantly, earlier studies allude to an effective *in vivo* antitumor immune response following passive transfer of antigen-loaded splenic or bone marrow derived-DCs, or administration of Flt3L[13,14]. Indeed, DCs seem to have the essential properties required for APCs to react as potent immunotherapeutic agents: migration and homing, antigen uptake, processing and presentation, and costimulation of lymphocytes[1].

For expression of antigenic peptide-MHC complexes on the cell surface, the peptides from endocytosed antigens need to be efficiently loaded onto newly synthesized class II molecules in compartments of the endocytic pathway[15]. Recent findings have emphasized that although immature DCs are active in the biosynthesis of MHC class II molecules and in peptide loading, the assembled peptide–MHC complexes accumulate in endosomes and lysosomes[16,17].

In EBV transformed-B lymphocytes, MHC–peptide complexes also accumulate in endosomes and lysosomes[18]. Raposo *et al.* showed that these compartments contain MHC class II-enriched internal vesicles that could be released outside the cell following direct fusion of the external membrane of the endosome with plasma membrane[19]. These vesicles, named exosomes, were shown to stimulate CD4+ T cell clones *in vitro*[19].

Here we examine the possibility that in addition to cell-to-cell contacts and cytokine/chemokine production, DCs may also trigger tumor-specific T-cell responses through the secretion of antigen presenting vesicles. We find that both multivesicular late endosomes and exosomes produced by DCs bear MHC class I and II molecules. Tumor peptide-loaded DC-derived exosomes induced CTL priming *in vivo* and suppressed growth or induced complete regression of several established murine tumors. DC-derived exosomes represent a novel cell-free therapeutic cancer vaccine.

MHC class I and II-containing exosomes

Peptide loading onto MHC class II molecules in human B lymphocytes occurs in late endocytic compartments called MHC class II compartments (MIICs). Using electron microscopy, two types of MIICs were distinguished morphologically: those containing numerous internal vesicles (multivesicular MIICs) and those displaying electron-dense concentrically arranged membrane sheets (multilamellar MIICs)[18]. Both types of MIICs contain lysosomal resident proteins such as Lamp1 and 2, HLA-DM, and several members of the tetraspan protein family, including CD63 and CD82 (unpublished results).

Surprisingly, using immunoelectron microscopy we found that in human monocyte derived (MD)-DCs, multivesicular MIICs also contain abundant MHC class I molecules (Fig. 1*a*). MHC class I and II, CD63 and CD82, were all found in both the external membrane of the endosomes and the intraluminal 60–90 nm vesicles (Fig. 1*a* and data not shown). Multilaminar compartments were not labeled with anti-MHC class I antibodies (not shown). In contrast to CD63 or CD82, MHC class I molecules were also detected at the cell surface (Fig. 1*a*).

Multivesicular MHC class I-containing compartments were often observed in close apposition to the cell surface, suggesting their direct fusion with the plasma membrane. Consistent with

this possibility, 60–90 nm vesicles, abundantly labeled with anti-MHC class I and II (Fig. 1*b–e*), CD63 (Fig. 1*b*) and CD82 (Fig. 1*e*) specific antibodies, were often observed close to the outer side of the plasma membrane (Fig. 1*b–e*).

These vesicles were isolated from DCs' culture supernatants by differential ultracentrifugation[19] and analyzed by whole-mount immuno-electron microscopy. A homogeneous population of vesicles of 60–90 nm diameter was observed (Fig. 2*a*). Just as for the vesicles from the exocytic profiles (Fig. 1), over 90% of these vesicles were labeled with anti-CD63 (Fig. 2*a*) and anti-CD82 antibodies (not shown), as well as with anti-MHC class I and/or class II antibodies (Fig. 2*b* and *c*, respectively). Co-localization of MHC class I and class II molecules in DCs' endosomes was also seen in confocal microscopy (data not shown). Exosome preparations were apparently devoid of plasma membrane-derived vesicles (which would label MHC class I and II, but not CD63 or CD82), microsome constituents and apoptotic bodies. They were also free of retroviruses, which can be identified morphologically by this technique. Therefore, DCs secrete a population of endosome-derived membrane vesicles which can bear both MHC class I and II molecules.

The presence of MHC class I molecules in DC derived-exosomes prompted us to address their potential $CD8^+$ T-cell stimulatory capacity *in vitro*. The MART-1/MelanA peptide$_{(27-32)}$ pulsed-DC derived-exosomes were capable of specifically stimulating IFNγ production and to a lesser extent, proliferation of a MART-1/MelanA-specific HLA-A2 restricted CTL clone LT12 (ref. 20). However, T-cell stimulation induced *in vitro* by exosomes, although significant and reproducible, was much weaker than that triggered directly by specific peptide pulsed-DCs (data not shown).

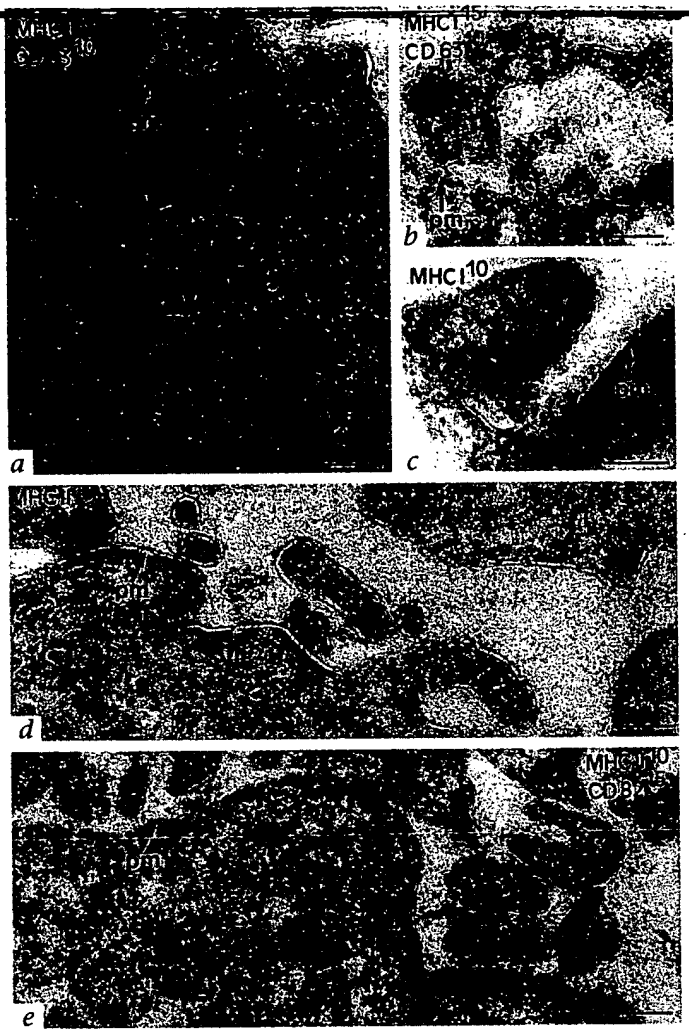

Fig. 1 Exocytosis of MHC class I- and II-positive exosomes in human MD-DCs. ***a–c***, Ultrathin cryosections of human DCs were single-immunogold-labeled with anti-MHC class I Heavy Chain (HC) antibodies and PAG15 (protein A gold) as indicated on the figure. MHC class I-positive 60–80 nm vesicles are observed close to the plasma membrane (pm). ***d–e***, Ultrathin cryosections were immunogold-labeled with an anti-class II antibody and PAG10 . Tetraspanins were revealed using an anti-CD63 antibody (*b*) and PAG10, or CD82 (*e*) and PAG 15. Bars: 200 nm.

Mouse DC-derived exosomes

To assess the capacity of DC-derived exosomes to induce effective T cell-mediated immune responses *in vivo*, we evaluated their antitumor effects in tumor-bearing mice. First, we determined if mouse DCs, like human DCs, produced exosomes. Bone marrow derived-DCs cultured in IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF) (BM-DCs)[21] were analyzed by confocal and electron microscopy and found to contain multivesicular late endosomes bearing both MHC class I and II molecules (data not shown). These DCs were pulsed with acid eluted tumor peptides and the supernatants harvested. Approximatively 15–25 μg of exosomes were routinely obtained from the supernatant of a 18–24 hour culture containing 5×10^6 BM-DCs propagated for 5–7 days in the presence of IL-4 and GM-CSF. These DCs expressed low levels of B7.2, CD40, MHC class I and II molecules, as assessed by flow cytometry analysis. Upon LPS stimulation, the expression of these molecules was upregulated. The markers expressed by the BM-DC derived-exosomes were characterized and quantified by electronic microscopy (data not shown) and western blotting.

MHC class I and II as well as CD86 and transferrin receptor (TfR) were found on exosomes (Fig. 3), and CD86 were enriched in exosomes, compared with the cell lysates. In contrast, although detected in the cell lysates, H2-M, Ii chain and calnexin (an endoplasmic reticulum specific marker) were undetectable in the exosomal preparations (Fig. 3). The exosomes from mouse BM-DCs were morphologically similar to the human DC-derived exosomes (by immunoelectron microscopy, data not shown). However, the DC populations obtained under these conditions are not 100% homogeneous. To demonstrate that DCs themselves are capable of producing exosomes, we used the well characterized murine DC line D1 (ref. 22). Immature D1 cells displayed abundant MHC class I and II containing multivesicular endosomes (as observed in confocal and immunoelectron microscopy) and produced exosomes, with morphology and markers similar to those produced by BM-DCs (data not shown).

Exosomes induce tumor growth suppression

BM-DCs cultured in IL-4 and GM-CSF and loaded with acid-eluted tumor peptides have been shown to mediate specific antitumor immune responses[21,23]. We investigated whether the exosomes produced by these DCs also displayed antitumor activity *in vivo*. Two experimental tumor models were chosen: P815 is an immunogenic but very agressive mastocytoma, syngeneic of DBA/2 (H-2^d), for which very few effective immunotherapies on day-ten-established tumors have been reported; and TS/A is a poorly immunogenic, spontaneous mammary carcinoma, expressing lower levels of MHC class I molecules, syngeneic to BALB/c (H-2^d). Acid-eluted tumor (AEP-P815 or AEP-TS/A) pep-

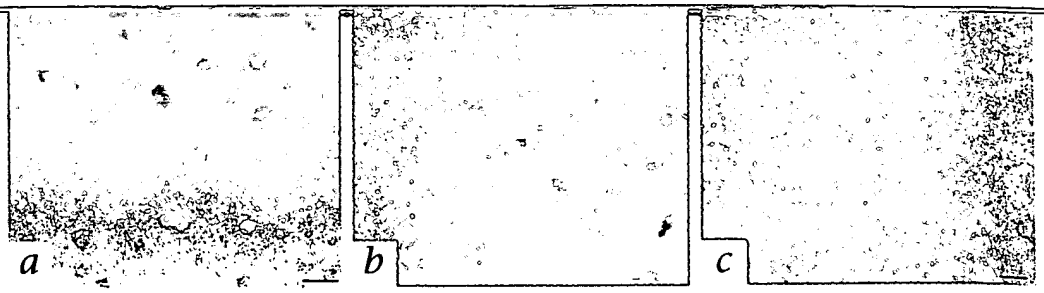

Fig. 2 Whole-mount immuno-electron microscopy of exosomes from human DCs. Exosome preparations contain small 50 to 90 nm vesicles labeled with an anti-CD63 antibody (*a*). A variable proportion of these CD63-positive vesicles (PAG 10) are also labeled with anti-class I HC antibodies (PAG 15) (*b*) and anti-class II antibodies (PAG 15) (*c*). Bars: 250 nm.

tides were pulsed onto syngeneic mouse BM-DCs as described[23] and exosomes were prepared from the DC culture supernatants and used for *in vivo* immunization.

Treatment of day-ten-established P815 tumors (50–90 mm^2 in size) involved a single intradermal (i.d.) administration of 3–5 μg of exosomes/mouse, corresponding to the exosomes produced by 5–10 × 10^5 tumor peptide-pulsed DCs in 18 hours. Within a week, tumor growth stopped in mice treated with exosomes derived from autologous tumor peptide pulsed DCs, and 40–60% of mice were tumor-free at day 60 (Fig. 4*a*). These animals had a long-lasting immune response, rejecting a lethal tumor challenge with P815 but not with the syngeneic leukemia L1210 (data not shown). Mice immunized with exosomes derived from self splenic peptide-pulsed DC showed no effect on tumor growth (Fig. 4*a*). Therefore, P815 peptide-pulsed DC derived-exosomes promote at least significant retardation of tumor growth in most animals. To demonstrate that exosome-induced immune responses are not simply due to direct effect of acid-eluted tumor peptides, day-five BM-DCs derived from H-2^d (DBA/2) or H-2^b (C57BL/6) mice were pulsed in parallel with acid eluted P815 tumor peptides. Exosomes were then isolated and injected i.d. into DBA/2 mice bearing 6–10-day established P815 tumors. As shown in Fig. 4*b*, only the syngeneic tumor peptide bearing exosomes induced tumor rejection (with up to 60% of mice tumor-free), whereas the allogeneic counterparts did not promote significant antitumor effects.

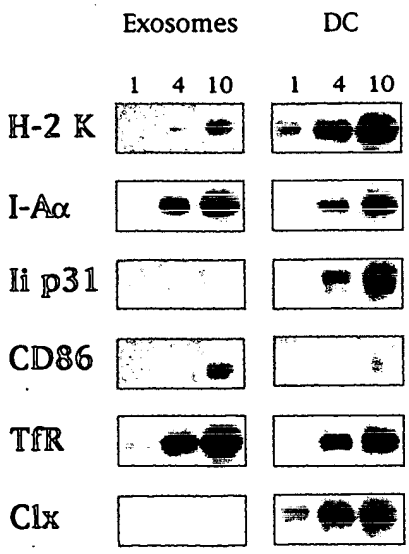

Fig. 3 Characterization of exosomes derived from mouse BM-DCs. Different amounts of exosomes (1, 4, 10 μg) obtained by differential ultracentrifugation of BM-DC culture supernatants were analyzed by Western Blotting for the presence of MHC class I (H-2K), class II (I-Aα), invariant chain (Ii p31), CD86, transferrin receptors (TfR) and calnexine (Clx). Similar amounts of proteins from the BM-DC lysates were analyzed in parallel. The molecular patterns of exosome preparations were similar in different BM-DC cultures from various strains of mice.

Similar antitumor effects were achieved with day-3–4-established TS/A tumors. In this setting, all mice had statistically significant delays in tumor growth that prolonged their survival (Fig. 4*c*). Preliminary results show that lung metastases were not detectable by two pathologists in d40 exosome treated mice (data not shown). These antitumor effects were not found in athymic Nu/Nu counterparts injected in parallel with the same exosome preparations (Fig. 4*d*), indicating that T cells are required for the exosome-induced antitumor immune responses.

In addition, exosomes directly primed tumor-specific CTL responses in P815 tumor-bearing hosts. Splenocytes from mice that rejected P815 tumors following immunization with exosomes were harvested at day 90 and cultured for five days in the presence of irradiated B7.1-expressing P815 cells to enhance specific precursor frequency. These effector cells were tested in a four-hour ^{51}Cr release assay against the autologous tumor cells P815 (H-2^d), against the irrelevant H-2^d leukemia L1210, and against the NK-cell target, YAC-1 cells. Significant specific lytic activity against P815 was achieved in splenocytes from exosome-immunized mice (Fig. 5). Interestingly, none of the spleens from littermates spontaneously rejecting P815 or bearing growing P815 tumors displayed cytolytic activity against P815 under the same conditions (data not shown). Therefore, a single injection of exosomes derived from DCs pulsed with the relevant peptides efficiently primes specific antitumor CTL responses *in vivo*.

Exosomes can substitute for DCs

Since adoptive immunotherapy using DCs has been reported, it is of interest to compare the relative efficacy of the novel cell-free vaccination using exosomes with that of whole DC preparations. We have already demonstrated using single tumor peptide epitope[21] or acid-eluted unfractionated tumor peptides[23], that BM-DCs cultured in GM-CSF and IL-4 (immature DCs) induce superior antitumor effects compared with DCs propagated in GM-CSF alone or GM-CSF and TNFα (mature DCs) in prophylaxis and therapy studies in tumor-bearing hosts. Moreover, preliminary data demonstrated that immature (IL-4 and GM-CSF) DCs contain and secrete many exosomes while mature DCs (TNFα and GM-CSF) produce few exosomes (unpublished data). Consequently, we compared the *in vivo* antitumor effects of exosomes derived from immature (GM-CSF and IL-4) BM-DCs with those mediated by whole DCs. Two groups of mice bearing established d9 P815 tumors were treated in parallel with 3–5 μg i.d./mouse of exosomes derived from 5–10 × 10^5 tumor peptide-loaded DC or with the 5–10 × 10^5 tumor peptide-loaded DCs themselves. In this particular day-ten tumor model, injection of DCs marginally affected tumor growth (20% of mice were tumor-free at day 60). In contrast, injection of exosomes delayed tumor growth and induced complete tumor regression in 60% mice.

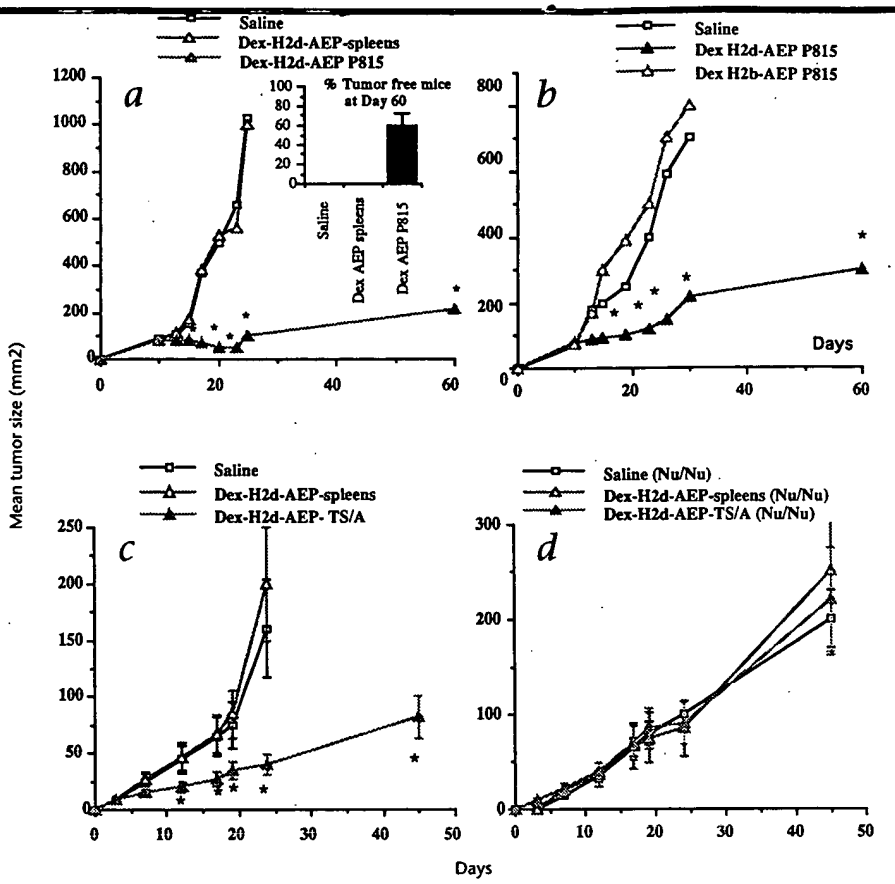

Fig. 4 Antitumor effects following administration of exosomes from acid-eluted tumor peptide (AEP)-pulsed BM-DCs. ***a***, DBA/2 mice bearing 50–80 mm^2 P815 tumors were immunized intradermally in the lower part of ipsilateral flank with exosomes (3–5 μg per mouse) from BM-DC H-2^d pulsed with acid-eluted P815 tumor peptides (DexH-2^d-AEP-P815) or with acid-eluted splenic peptides (DexH-2^d AEP-spleens) from DBA/2 mice. Tumor sizes were monitored twice a week. The inset depicts the percentage of tumor-free mice at day 60. ***b***, DBA/2 mice bearing P815 tumors (as described in *a*) were treated with exosomes prepared from BM-DCs from either DBA/2 mice (Dex H-2^d AEP-P815) or from C57BL/6 mice (Dex H-2^b -AEP-P815), pulsed with acid-eluted P815 tumor peptides. Only the syngenic exosomes induced a delay in tumor growth. ***c***, The exosome-mediated antitumor effects are also observed in a poorly immunogenic model (TS/A) and are T cell-dependent. At days 3–4, exosomes (5 μg/mouse) from BM-DC (H-2^d) pulsed with acid-elutedTS/A tumor peptides (Dex H-2^d-AEP-TS/A) or with splenic peptides (Dex H-2^d-AEP-Spleens) of BALB/c mice were administered intradermally in the lower ipsilateral flank of BALB/c mice. ***d***, In parallel with the experiment shown in *c*, nude BALB/c mice were injected under the same conditions with the same exosome preparations. No antitumor effects were observed in nude mice. Asterisks represent significant results at 95% using Fisher's exact method compared with injection of saline or splenic peptide-pulsed DC-derived exosomes. All of these experiments were reproduced at least twice with similar results.

Similar experiments were performed with established TS/A tumors in BALB/c animals. In this case, as before[23], DCs induced only a significant delay in tumor growth. However, in this tumor model as well, the effects of exosomes on tumor growth were more significant compared with those mediated by DCs (not shown). Therefore, exosomes produced in high amounts by immature DCs could substitute for dendritic cell therapy in the treatment of established tumors.

Discussion

We have demonstrated that a single i.d. injection of tumor peptide-loaded DC-derived exosomes induces a potent immune response resulting in tumor growth delay or complete tumor eradication. These antitumor effects were T-cell mediated and most likely involved $CD8^+$ CTLs since no antitumor effect induced by exosomes was observed in nude mice, the effects of exosomes bearing allogeneic MHC molecules were not significant, and specific antitumor CTL activities were detected in the spleen of cured animals.

Cell-free cancer vaccines as effective as cellular vaccines for therapy of established tumors have not been reported so far. P815 is considered an immunogenic tumor and effective prophylaxis in this model has been reported[24]. However, P815 is also very aggressive, and established 60–80 mm^2 tumors are difficult to eradicate, even with whole DCs. Actually, no efficient therapeutic vaccine administered outside the tumor site in established P815 tumors has been reported. TS/A is considered a poorly immunogenic, spontaneously metastatic tumor model[25], where unfractionated tumor peptide pulsed DC-based immunotherapy has marginal efficacy.

Why should exosomes be more effective than the immature DCs from which they derive? Several hypotheses can be suggested. First, it is known that tumor cells induce immunomodulatory events in the host. In a tumor environment, DCs display phenotypic modifications, such as downregulation of costimulatory molecules, that impair their antigen presenting functions[26]. *Ex vivo*, expanded DCs represent valuable immunotherapeutic options for cancer-bearing patients, but phenotypic and functional changes (such as surface markers, migration pathways and T-cell stimulation capacities) can be anticipated following withdrawal from the culture medium[27]. Cell-free antigen presenting vesicles should be unaffected by these factors. Also, since lipid compounds have been reported to have adjuvant qualities[28], the lipid composition of exosomes may boost their stimulatory capacities. The simultaneous presence of both MHC class I and II, together with high levels of costimulatory molecules (CD86) (Fig. 3) may also contribute to their *in vivo* effects. The significant enrichment for several members of the tetraspan protein family in exosomes, including CD63 and CD82, also suggests that these proteins are involved in the potent *in vivo* immunostimulatory effects of exosomes. Finally, in contrast with MHC class II molecules, the half-life of MHC class I molecules is not prolonged on the cell surface upon DC maturation[17]. Therefore, it is likely that following DC injection, the preformed MHC class I-peptide complexes are not exposed any longer on the DC surface, limiting the CD8+ specific T cell responses *in vivo*. Exosomes are 60–90 nm membrane vesicles that carry in their membrane functional MHC class I and II molecules with their peptide binding domain oriented towards the extracellular milieu[19,29]. Exosomes inefficiently, but significantly and reproducibly, stimulate T cells

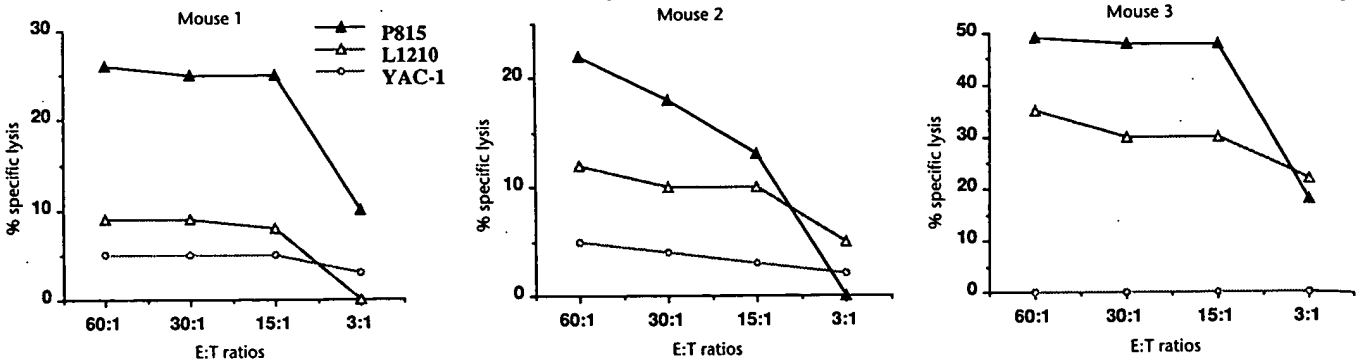

Fig. 5 DC-derived exosome-based vaccines prime tumor-specific CTL *in vivo*. Following *in vitro* stimulation, the cytotoxic activity of splenocytes from mice that rejected their established P815 tumors after exosome treatment were tested in a ^{51}Cr-release assay against chromium-labeled target cells P815 (autologous tumor cells), an H-2^d–irrelevant leukemia L1210 or the NK-sensitive YAC cell line. Specific lysis is depicted for each of the three representative mice (12%–50% lysis was achieved). Mice that underwent P815 spontaneous regressions and P815-bearing mice were tested in parallel, and no cytotoxicity was found (data not shown). The experiment has been reproduced twice with similar results.

in vitro (class I restricted CD8$^+$ T cells or allogeneic CD4$^+$ T cells (data not shown)). Exosome preparations could be contaminated with adjuvant components from fetal bovine serum and/or with unbound tumor peptides. However, exosomes from H-2^b derived DCs treated under the same conditions (which should therefore contain the same putative contaminants) did not mediate any antitumor effects in H-2^d mice. Nethertheless, *in vitro* results tend to argue against a direct stimulation of T cells to account for the efficient T-cell priming observed *in vivo* in tumor-bearing hosts.

The potent *in vivo* immunostimulatory effects of exosomes suggest a physiological role of natural microparticulate liposome-like vectors for communication between cells of the immune system. The striking observation is that immature DCs, purportedly considered to be poor antigen presenting cells[30], are actually capable of secreting antigen presenting exosomes that efficiently prime T-cell responses *in vivo*. Importantly, we found that production of exosomes by DCs is a regulated process. Indeed, the amount of exosomes produced by the mouse DC D1 line or BM-DCs was reduced following maturation. In contrast, the basal secretion of exosomes can be further and significantly enhanced (at least fivefold) by incubating the immature DCs with cytokines (such as IL-10 or IFNγ) or by transiently lowering the pH (manuscript in preparation).

It was unexpected, however, that multivesicular late endosomes and exosomes in DCs bear MHC class I molecules. It is most likely that the endosomal MHC class I molecules are delivered to the endocytic pathway after internalization from the cell surface[17]. Cycloheximide treatment of B and dendritic cells indicates that these endosomal class I molecules, located in multivesicular bodies and MIICs, are derived from the plasma membrane (M. Kleijmeer, pers. comm.). It is also unexpected to find transferrin receptors (TfR) in exosomes. Indeed, in EBV-transformed B lymphocytes, multivesicular late endosomes do not express TfR[19]. However, the distribution of TfR may vary between cell types and preliminary results indicate that in mouse BM-DC, TfR is found in multivesicular endosomes (unpublished results). This is not surprising given the high endocytic activity of immature DCs. However, the presence of TfR may in part be due to contamination of our exosome preparations with plasma membrane or early endosomes.

Even though exosome release has been associated with clearance of TfR, reticulocyte maturation and differentiation into an erythrocyte pathway[31], the physiological role of exosome secretion and function *in vivo* is still a matter of debate. Our results indicate that exosome secretion by DCs may result in T-cell stimulation in the absence of direct contact between

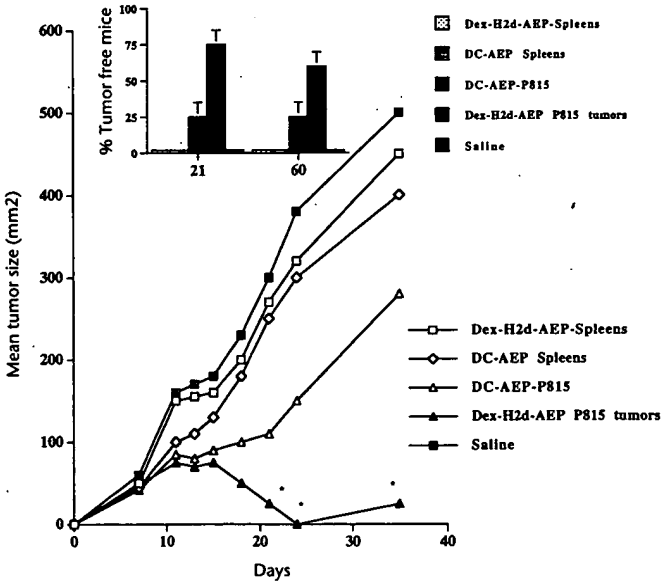

Fig. 6 Exosomes derived from immature DCs can substitute for DCs in eradication of established tumors *in vivo*. 5–10 × 10^5 immature BM-DCs (IL-4 and GM-CSF) pulsed with either acid–eluted splenic peptides (open diamands) or P815 tumor peptides (open triangles) were administered intradermally in the ipsilateral inguinal area at day 9 following P815 establishment. In parallel, the supernatants of other BM-DCs (pulsed in a similar way) were harvested following an 18 hour incubation with splenic peptides (open squares, Dex-H2^d-AEP-spleens) or with P815 tumor peptides (solid triangles, Dex-H2^d-AEP-P815), ultracentrifuged and the exosome pellets distributed in PBS and injected intradermally in the ipsilateral inguinal area, in five mice. Closed squares, saline-treated animals. A single administration of either exosomes or whole immature BM-DCs was done at day nine. Tumor size was measured biweekly. Inset represents the percentage of tumor-free mice immunized against P815 at day 21 and day 60. Representative data are shown for two similar experiments. Asterisks indicate significant results at 95% using Fisher's exact method, as compared with injection of saline or of peptide-pulsed DCs.

the antigen presenting cell and the responding T cell. Nevertheless, the physiological role of DC derived-exosomes in priming T-cell immune responses *in vivo* remains unclear. It is conceivable that T-helper cytokines are delivered to the DC upon arrival in the lymph node T-cell enriched areas. Antigen presenting vesicles would then be released to amplify specific T-cell clonal expansion. Alternatively, other host APCs could take up these exosomes to transport such antigenic vesicles to specific sites where priming of naive T cells and/or B-cell crosstalk could be elicited.

Our results strongly support the implementation of DC-derived exosomes for cancer immunotherapy. As cancer vaccines, exosomes combine the advantages of DCs (high levels of peptide bound-MHC class I and II molecules along with T-cell costimulation) with those of cell-free vectors. However, the clinical application of exosomes will require extensive biochemical characterization and analysis of the mechanisms underlying their bioactivities.

Methods

Exosome isolation. DCs were incubated 18–20 hours with acid-eluted tumor peptides (prepared as described[23]). DCs supernatants were harvested, centrifuged (at 4 °C), at 300 *g* for 20 min and then at 10 000 *g* for 30 min (to eliminate cell debris). Exosomes were then pelleted at 100,000 *g* for one hour, and washed once in a large volume of PBS (over 100-fold the final volume of resuspension of the exosomes). The protein concentrations in exosome preparations were measured by Bradford assay (Biorad). The slightly acidic pH transiently induced by the acid peptide elutions increased the amounts of exosomes produced by DCs. Three to five µg of exosome were routinely obtained from 5–10 × 10^5 DCs in 18–20 hours.

Electron microscopy. Immunogold labeling on ultrathin cryosections: dendritic cells were fixed, processed for ultracryomicrotomy and immunogold as described[29]. Immunogold labeling on isolated exosomes : pellets obtained after ultracentrifugation at 100,000 *g* were fixed with paraformaldehyde 2% in PB and loaded onto EM grids. The immunogold labeling, contrasting and embedding procedures were carried out as described for ultrathin cryosections[19]. The anti-human class I antibody was kindly provided by H. Ploegh[32] and anti-CD63 mAb binds a lysosomal membrane glycoprotein as described by Metzelaar *et al.*[33]. The anti-CD82 antibodies were provided by H. Conjeaud (Institut Cochin, Paris).

Western blots. The 1–4 and 10 µg of exosome proteins or of total cell lysates were solubilized in Laemli sample buffer at 95 °C, with reducing conditions, and separated by SDS-PAGE. Proteins were transferred to PVDF filters (Amersham) and detected by Western blot using anti-MHC class II (rabbit serum anti-I-Aα), anti-MHC class I (rabbit serum anti-H-2-K), rat anti-invariant chain Ii p31 (In1), rat anti-CD86 (GL1, Pharmingen), mouse anti-transferrin receptor TfR (H68-4) or rabbit serum anti-calnexin (gift from Ari Helenius).

Mice and tumor cell lines. DBA/2J (H-2^d) and BALB/c (H-2^d) female mice 6–8 weeks of age were purchased from the Iffa Credo Laboratories (Lyon, France) and raised in pathogen-free conditions. Nude mice were kept in protected microenvironments. P815 (H-2^d) is a methylcholanthrene-induced mastocytoma, syngeneic with DBA/2, provided by T. Boon (Ludwig Institute, Belgium). TS/A (H-2^d) is a spontaneously-arising undifferentiated mammary adenocarcinoma, syngeneic with BALB/c, provided by G. Forni (Immunogenetic and Histocompatibility Center, Turin, Italy). All tumor cell lines were maintained in RPMI 1640 supplemented with 10% endotoxin-free fetal calf serum (Gibco BRL), 2mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, essential aminoacids and pyruvate.

Experimental mouse models. Twice the minimal tumorigenic dose of tumor cells (5 × 10^5 P815, 10^5 TS/A) was inoculated intradermally in the upper right flank of DBA/2 and BALB/c mice, respectively. Animals with established tumors at days 3–4 for TS/A, or days 8–10 for P815, were immunized with a single intradermal injection of 3–5 µg of exosomes per mouse in the lower ipsilateral flank. These procedures were similar for both immunocompetent and Nude mice. The tumor size was monitored biweekly and mice were sacrifized when bearing ulcerated or huge tumor burdens. All experiments were performed two to three times using individual treatment groups of five mice per group.

Cytotoxicity assays. Splenocytes from DBA/2 mice were harvested and 30 × 10^6 cells were cocultured with 3 × 10^6 10,000-rad-irradiated P815 cells transfected with the B7.1-encoding cDNA (provided by P. Kourilsky, Institut Pasteur, Paris, France) for 5 days at 37 °C, 5% CO_2. The P815, L1210 or YAC-1 cells were labeled in CM containing $Na_2{}^{51}CrO_4$ (150 µCi per million cells) for 1–2 hours at 37 °C. Equal volumes (100 µl) of target and effector cells (that is, splenic cells after 5 day-*in vitro* restimulation of effector to ^{51}Cr-labeled-target ratios of 60:1, 30:1, 15:1 or 3:1) were plated in triplicate round-bottomed microtiter wells for 4 hours at 37 °C and 5% CO_2. After a 4-hour incubation, 100 µl of supernatant was collected, and the percentage of specific ^{51}Cr release was calculated using the formula percent release = 100 × (cpm experiment–cpm spontaneous release) / (cpm maximum release–cpm spontaneous release), where spontaneous release was that obtained from target cells in CM alone, and maximum release was obtained from target cells incubated in 5% Triton X-100 (Sigma Chemical Co.).

Dendritic cell cultures. Mouse BM-DCs were prepared as described[21] in CM with IL-4 and GM-CSF (1000 IU/ml each) and pulsed with acid-eluted tumor peptides[23,34]. The floating DCs expressed CD11b, CD11c, CD86, DEC205, MHC class I and II and CD40. They were negative or low for CD3 and B220 expression. Human-monocyte-derived DCs were obtained from the adherent fraction of mononuclear cells of healthy volunteers and were incubated 7–8 days in AIMV containing L-Glu, antibiotics and rh IL-4 and rh GM-CSF (1000 IU/ml each, Schering Plough, Kenilworth, NJ, USA). After 8 days in culture, the loosely adherent or floating cells had typical dendritic morphology, expressed high levels of MHC class I and II molecules, CD40 and CD86 (ref. 35); most were positive for CD1a and CD11c but low or negative for CD2, CD3, CD14, CD19 and CD83.

Statistical analyses. Fisher's exact method was done to interpret the significance of differences between experimental groups (presented as mean +/- SEM). Significance at 95% confidence limits is presented for individual experiments.

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